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Aspergillus Polymerase Chain Reaction: Systematic Review of Evidence for Clinical Use in Comparison With Antigen Testing

P. Lewis White,¹ John R. Wingard,² Stéphane Bretagne,³ Jürgen Löffler,⁴ Thomas F. Patterson,⁵ Monica A. Slavin,⁶ Rosemary A. Barnes,⁷ Peter G. Pappas,⁸ and J. Peter Donnelly⁹

¹Public Health Wales, Microbiology Cardiff, United Kingdom; ²University of Florida Health, Gainesville; ³Paris Diderot University, Saint Louis Hospital-AP-HP, France; ⁴Wuerzburg University, Germany; ⁵University of Texas Health Science Center and South Texas Veterans Health Care System, San Antonio; ⁶Peter MacCallum Cancer Centre, University of Melbourne, Australia; ⁷Infection, Immunity and Biochemistry, Cardiff University, United Kingdom; ⁸University of Alabama at Birmingham; and ⁹Radboud University Medical Centre, Nijmegen, The Netherlands

Background. *Aspergillus* polymerase chain reaction (PCR) was excluded from the European Organisation for the Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) definitions of invasive fungal disease because of limited standardization and validation. The definitions are being revised.

Methods. A systematic literature review was performed to identify analytical and clinical information available on inclusion of galactomannan enzyme immunoassay (GM-EIA) (2002) and β -D-glucan (2008), providing a minimal threshold when considering PCR. Categorical parameters and statistical performance were compared.

Results. When incorporated, GM-EIA and β -D-glucan sensitivities and specificities for diagnosing invasive aspergillosis were 81.6% and 91.6%, and 76.9% and 89.4%, respectively. *Aspergillus* PCR has similar sensitivity and specificity (76.8%–88.0% and 75.0%–94.5%, respectively) and comparable utility. Methodological recommendations and commercial PCR assays assist standardization. Although all tests have limitations, currently, PCR is the only test with independent quality control.

Conclusions. We propose that there is sufficient evidence that is at least equivalent to that used to include GM-EIA and β -D-glucan testing, and that PCR is now mature enough for inclusion in the EORTC/MSG definitions.

Keywords. *Aspergillus*; PCR; galactomannan; β -D-glucan; aspergillosis.

Polymerase chain reaction (PCR) to aid diagnosis of invasive aspergillosis (IA) has been used for over 2 decades, but is not included in the European Organisation for the Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) definitions of invasive fungal disease. In the 2002 definitions, PCR was excluded

on the basis of potential false-positive results and lack of standardized commercial testing platforms [1]. It was reconsidered when the definitions were revised in 2008, but despite continued development, molecular methods were not to be included because there was no standard; and clinically, validation was limited [2].

The EORTC/MSG definitions are currently under review. To provide impartial review of *Aspergillus* PCR technology, we reassessed information that was available at the time of inclusion of previous biomarkers, galactomannan enzyme immunoassay (GM-EIA) in 2002, and β -D-glucan (BDG) in 2008. This baseline information provides a systematic dataset for categorical and standardized comparison of PCR, and any other emerging or future diagnostic assays (eg, *Aspergillus* lateral flow device; [3, 4]). The evidence forms the basis for full review of manuscripts describing *Aspergillus* PCR allowing the EORTC/MSG consensus committee to consider PCR in future definitions.

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Correspondence: P. Lewis White, PhD, Public Health Wales, Microbiology Cardiff, University Hospital of Wales, Heath Park, Cardiff CF14 4XN, UK (lewis.white@wales.nhs.uk).

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This manuscript describes the findings of this initial review and is a joint effort of the European *Aspergillus* PCR initiative (EAPCRI), a working group of the International Society for Human and Animal Mycology, the EORTC Infectious Disease Group, and the Mycoses Study Group (MSG).

REVISION PROCESS

Evaluating the GM-EIA and BDG Evidence Available Prior to Incorporation Into Disease Definitions

Manuscripts describing the use of GM-EIA and BDG were identified by literature searches using Medline/PubMed using the following keywords: *Aspergillus* diagnosis, galactomannan EIA or ELISA [enzyme-linked immunosorbent assay], *Aspergillus* antigen, aspergillosis for GM-EIA, glucan, β -glucan, fungal infection (diagnosis), mycoses, *Aspergillus* for BDG. To confirm the quality of the literature searches and identify missed articles, bibliographies of representative meta-analyses were screened [5–12]. Only manuscripts published before incorporation of both assays into the EORTC/MSG definitions were included.

The focus of studies was determined by reviewing the title and abstract. If the study was a technical description or an animal model evaluation, it was assessed for any potential useful information (eg, cross-reactivity with other fungal species, interassay reproducibility, or potential kinetics of antigen release) but excluded from performance analysis. For clinical evaluation, factors deemed important with respect to diagnostic use were identified (Table 1). As the BDG assay detects a broader range of fungi, performance data specific to the detection of IA were retrieved from the original manuscript, and manuscripts were excluded if this was not possible.

Evaluating the Evidence Base for *Aspergillus* PCR Testing

Manuscripts describing the use of PCR were identified by literature searches using PubMed. More than 200 manuscripts were available for review [13, 14]. Many were available before the last EORTC/MSG revision, so the focus was on important developments in the field of *Aspergillus* PCR, particularly after 2008. Relevant meta-analyses were used, representing an independent and statistically sound representation of clinical performance [15–19].

Statistical Analysis

Sensitivity, specificity, positive and negative predictive values, likelihood ratios, and diagnostic odds ratios were calculated. Prospective and retrospective studies were analyzed both separately and combined. The 95% confidence intervals (CIs) were calculated for proportions, and when comparing parameters between different assays, Fisher exact test was used to generate a 2-sided *P* value, with a value of $\leq .05$ deemed significant.

EVIDENCE FOR ANTIGEN TESTING

GM-EIA: Manuscript Characteristics

Sixty-one articles were retained for further review. Sixteen were subsequently excluded as they did not describe the use of the BioRad GM-EIA or a precursor of this test (eg, latex agglutination or in-house ELISA tests). A breakdown of the remaining manuscripts is shown in [Supplementary Figure 1](#).

GM-EIA: Analytical Evidence

Six manuscripts evaluated GM-EIA detection and provided information pertinent to clinical performance. Cross-reactivity with non-*Aspergillus* fungal species was determined ([Supplementary Table 1](#)) [20]. Of greater concern was the generation of false-positive results (8/40) associated with bacteremia and candidemia in febrile neutropenic patients [20].

Intra and interassay reproducibility of the GM-EIA was investigated. Although the qualitative agreement (positive/negative) between centers was excellent, the quantitative inter-assay precision was only 25.6% [21].

GM-EIA: Preinclusion Clinical Performance

Of the 26 articles describing clinical performance, 17 were prospective validations. Fifteen of the prospective studies described the performance of testing serum from 2067 adults with an IA incidence of 13.9% [22–36]. Six of the retrospective studies described performance testing serum from 214 adults with an IA incidence of 25.7% [37–42]. Neither sensitivity nor specificity differed significantly between prospective or retrospective studies ($P = .57$ and $P = .45$, respectively). The performance of serum GM-EIA preincorporation into the EORTC/MSG definitions is summarized in Table 2.

Three studies described the use of GM-EIA in 458 pediatric patients, with an IA incidence of 5.5% [25, 43, 44]. The pooled sensitivity and specificity were 100% (95% CI, 86.7%–100%) and 90.1% (95% CI, 86.8%–93.2%), respectively. The testing of bronchoalveolar lavage (BAL) samples was limited, with pooled sensitivities and specificities of 89.5% (95% CI, 68.6%–97.1%) and 93.3% (95% CI, 81.4%–97.6%), respectively [31, 42]. Evidence for GM-EIA testing of cerebrospinal fluid was limited to case reports or small series [35, 45–48].

Characteristics and Clinical Utility of GM-EIA Before 2002

A summary of GM-EIA characteristics is described in Table 1. Methodological standardization was evident, as only one commercial assay was available. However, since the original EORTC-MSG definitions, the analytical threshold for positivity has been reduced from an optical density (OD) index of 1.5 to 0.5 [21, 23–24, 49–50]. The choice of optimal threshold will be defined by the strategy chosen. For highly sensitive screening, a lower OD index ≤ 0.5 is preferable, whereas to provide accurate

Table 1. Assay Characteristics With the Potential to Influence Diagnostic Utility and Fungal Biomarker Information Available at the Time of Incorporation Into the European Organisation for the Research and Treatment of Cancer/Mycoses Study Group Definitions

| Characteristic | Description | GM-EIA (<2002) | β -D-Glucan (<2008) |
|--------------------------------|--|---|--|
| Methodological standardization | A standard method is available for performing the assay and interpreting the result. | Single commercial assay with SOP. | Four different commercial assays available utilizing different methods and materials. |
| Control material | Material is available to determine whether the assay is performing efficiently and individual experimental runs are valid. | Controls supplied by the manufacturer. No independent material available. | Controls supplied by the manufacturer. No independent material available. |
| Quality control | Independent (external) QC schemes are available to determine performance of assays at individual centers. | No manufacturer or independent QC available | No manufacturer or independent QC available |
| Clinical validity | Assay performance (sensitivity/specificity etc) in defined cases/controls. An understanding of the causes of false-positive and false-negative results. | Good performance when testing serum from hematology/BMT populations (Table 2) [22–31, 34–38, 40–42]. Limited evaluations for the testing of BAL or CSF samples, and pediatrics/neonates or CGD, HIV liver transplant patients [25, 29, 31, 32, 35, 36, 39–47, 93]. False Positives – Yes False Negatives – Yes | Good clinical performance for the diagnosis of IA when testing serum/plasma from hematology patients (Table 2) [51, 53, 55, 57, 58, 60, 62, 63]. Limited evidence for use in other conditions [58, 59, 63]. False positives – Yes False negatives – Yes |
| Clinical utility | Assays have been incorporated into strategically constructed care pathways, and have a direct influence on patient management and potential effect on local hospital policy. | Limited and often conflicting, with no strategic studies investigating utility were available. | Limited and often conflicting, with no strategic studies investigating utility were available. |

Abbreviations: BAL, bronchoalveolar lavage; BMT, bone marrow transplantation; CGD, chronic granulomatous disease; CSF, cerebrospinal fluid; GM-EIA, galactomannan enzyme immunoassay; HIV, human immunodeficiency virus; IA, invasive aspergillosis; QC, quality control; SOP, standard operating procedure.

Table 2. Performance of Galactomannan Enzyme Immunoassay and β -D-Glucan When Testing Adult Serum for the Detection of Invasive Aspergillus Prior to Incorporation Into the European Organisation for the Research and Treatment of Cancer/Mycoses Study Group Definitions for Diagnosing Invasive Fungal Disease

| Parameter | Assay (Preincorporation Into EORTC/MSG Definitions) | | | | | |
|----------------------|---|-------------------|-------------------|-----------------------------------|-------------------|-------------------|
| | GM-EIA Testing (<2002) | | | β -D-Glucan Testing (<2008) | | |
| | Overall | Prospective | Retrospective | Overall | Prospective | Retrospective |
| Sensitivity (95% CI) | 81.6% (75.6–86.4) | 80.8% (74.2–86.1) | 85.5% (68.9–94.2) | 76.9% (66.7–84.8) | 66.1% (53.7–76.7) | 85.2% (75.9–91.3) |
| Specificity (95% CI) | 91.6% (89.9–93.1) | 91.8% (90.0–93.3) | 89.9% (82.0–94.7) | 89.4% (87.0–91.4) | 88.9% (86.5–90.9) | 90.2% (87.2–92.5) |
| PPV (95% CI) | 63.3% (57.3–69.0) | 61.4% (54.8–67.5) | 74.6% (58.3–86.2) | 44.7% (36.8–52.6) | 31.5% (22.1–42.8) | 59.5% (47.5–70.5) |
| NPV (95% CI) | 96.6% (95.3–97.5) | 96.7% (95.4–97.7) | 94.7% (87.8–97.9) | 97.2% (95.7–98.2) | 97.1% (95.1–98.1) | 97.3% (94.5–98.7) |
| Positive LR | 9.8 | 9.9 | 8.5 | 7.2 | 6.0 | 8.7 |
| Negative LR | 0.2 | 0.21 | 0.16 | 0.3 | 0.38 | 0.16 |
| DOR | 48.6 | 47.2 | 52.5 | 28.0 | 15.6 | 52.9 |

For galactomannan enzyme immunoassay, a total of 26 studies have been included, involving 2281 patients with an incidence of invasive aspergillosis (IA) of 15.0%, and a positivity index of 1.5. For β -D-glucan, a total of 13 studies have been included involving 1423 patients with an incidence of IA of 10.0%; patients diagnosed with other forms of invasive fungal disease have been excluded from the analysis. The positivity threshold varied between assays.

Abbreviations: CI, confidence interval; DOR, diagnostic odds ratio; EORTC/MSG, European Organisation for the Research and Treatment of Cancer/Mycoses Study Group; GM-EIA, galactomannan enzyme immunoassay; LR, likelihood ratio; NPV, negative predictive value; PPV, positive predictive value.

diagnosis, specificity can be improved with a higher OD index of 1.5. The specificity of GM-EIA as calculated by both meta-analyses (including studies using the lower threshold) is significantly lower than that calculated for studies performed before 2002 using an OD index ≥ 1.5 ($P < .0001$ – $.0003$) (Tables 2 and 3). In both meta-analyses, there was a clear trend of increased specificity using an OD index ≥ 1.5 [7, 8]. The US Food and Drug Administration has recently selected a higher threshold for positivity for diagnosing IA in clinical trials (invasive aspergillosis biomarker, [/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM420248.pdf](#)). Alternatively, a variable threshold for positivity could be used to provide confidence in both excluding disease and for accurate diagnosis, as recently described for BAL testing [51].

There were no quality control (QC) programs available to monitor performance before 2002. Both false-positive and false-negative results were identified, but assay specificity was improved when 2 positive results were required for significance, as recommended by the manufacturer (Table 1 and [Supplementary Table 1](#)).

Twice-weekly testing was strongly advocated, and there was evidence linking the GM-EIA index to the burden of disease and prognosis, although poor interassay precision may affect monitoring of disease progression and response to therapy [21]. The impact of GM-EIA on patient management was variable; some studies showed positive tests to be an early indicator of disease, whereas others found only a limited effect on patient management ([Supplementary Table 1](#)). No strategic studies investigating utility were available.

β -D-Glucan: Manuscript Characteristics

Fifty-three articles were retained for further review. Ten were excluded, as they were specific to the diagnosis of invasive candidiasis or *Pneumocystis jirovecii* pneumonia. Forty-three manuscripts were retained for detailed review, and a breakdown of their structure is shown in [Supplementary Figure 2](#).

β -D-Glucan: Analytical Evidence

In an in vitro study, the release of BDG occurred primarily during logarithmic growth (<24 hours), corresponding with increasing fungal biomass, albeit later than the release of GM [52]. As part of multicenter clinical studies, interlaboratory reproducibility of the BDG assay was shown to be good ($r^2 = 0.93$ – 0.99 ; interassay precision 9.1%), and BDG levels remained stable, with neither storage duration nor freezing/thawing having any measurable effect on performance [53, 54]. The analytical specificity of the Fungitec G-Test and Fungitell assays were confirmed, and cross-reactivity with non-glucans (eg, Xylan, Galactan) containing the β -1–3 linkage or glucans containing β -1–4 or β -1–6 linkage was excluded [55, 56]. The reactivity of the Fungitell assay was determined to be 2.5-fold less than the Fungitec-G assay, necessitating different analytical thresholds for positivity between assays [55]. There were no studies comparing the analytical performance of all BDG assays.

β -D-Glucan: Preinclusion Clinical Performance

Thirteen articles including 1423 patients, of whom 143 were diagnosed with proven/probable IA, were analyzed. Performance statistics for the diagnosis of IA are shown in Table 2.

Table 3. Performance of Fungal Biomarker Assays for the Detection of Invasive Aspergillosis as Determined by the Relevant Published Meta-analyses

| Parameter | Assay | | | | | | | | | | | | |
|----------------|------------------------------|------------------------------|-------------------------|-------------------------|----------------------------|---------------------------------------|-------------------------|-----------------------------|---------------------------|--------------------------|---------------------------|------------------------------|--------------------------------|
| | GM-EIA | | | | β -D-Glucan | | | | PCR | | | | |
| | Leeflang [7] ^a | Pfeiffer [8] ^b | Zou [5] ^c | Guo [6] ^d | Lamoth [9] ^e | Karageorgopoulos [10] ^f | He [11] ^g | Onishi [12] ^h | Tuon [15] ⁱ | Sun [16] ^j | Avni [18] ^k | Mengoli [17] ^l | Arvanitis [19] ^m |
| | Serum | | BAL | | Plasma/Serum | | | | BAL | | | Blood | |
| Sensitivity, % | 79.3 | 79.3 | 83.6 | 85.7 | 56.8 | 77.1 | 77.0 | 77.3 | 78.4 | 79.6 | 76.8 | 88.0 | 84.0 |
| Specificity, % | 80.5 | 86.3 | 89.4 | 89.0 | 97.0 | 85.3 | 81.3 | 83.4 | 93.7 | 94.1 | 94.5 | 75.0 | 76.0 |
| PPV, % | 30.3 | 46.9 | 64.4 | 66.7 | 55.6 | 30.2 | 28.7 | 33.4 | 74.1 | 79.2 | 79.9 | 36.7 | 41.3 |
| NPV, % | 97.3 | 96.5 | 95.9 | 96.0 | 97.1 | 97.8 | 97.3 | 97.2 | 94.9 | 94.2 | 93.4 | 97.4 | 95.9 |
| Positive LR | 4.06 | 5.81 | 7.89 | 7.81 | 18.90 | 5.24 | 4.12 | 4.65 | 12.43 | 13.38 | 13.87 | 3.52 | 3.50 |
| Negative LR | 0.26 | 0.24 | 0.18 | 0.16 | 0.44 | 0.27 | 0.28 | 0.27 | 0.23 | 0.22 | 0.25 | 0.16 | 0.21 |
| DOR | 15.8 | 24.2 | 43.0 | 48.6 | 43.0 | 19.4 | 14.7 | 17.2 | 53.8 | 61.7 | 56.52 | 22.1 | 16.6 |

Abbreviations: BAL, bronchoalveolar lavage; DOR, diagnostic odds ratio; GM-EIA, galactomannan enzyme immunoassay; LR, likelihood ratio; NPV, negative predictive value; PCR, polymerase chain reaction; PPV, positive predictive value.

^a Includes 87 proven/probable invasive aspergillosis (IA) cases and 814 controls where performance was reported using a 0.5 positivity index [7].

^b Includes 87 proven/probable IA cases and 571 controls where performance was reported using a 0.5 positivity index [8].

^c Includes 493 proven/probable IA cases and 2144 controls where performance was reported using a 0.5 positivity index. The results represent pooled parameters (sensitivity/specificity) and differ from the published data that were summary estimates as calculated by Summary Receiver Operator Characteristic (SROC) analysis (sensitivity 87% and specificity 89%) [5]. Due to limitations in the reporting of the number of cases and controls in studies specifically using a 0.5 positivity index, it was not possible to calculate the PPV/NPV from data in the original manuscript. Consequently, PPV/NPV have been calculated using the data from the original publications, using publications only listed in the meta-analysis of Zou et al [5].

^d Includes 133 proven/probable IA cases and 519 controls where performance was reported using a 0.5 positivity index and is based on pooled performance data, not summary estimates as described in the meta-analysis of Guo et al [6].

^e Includes 44 proven/probable IA cases and 653 controls where results are based on pooled performance data [9]. Performance in relation to the detection of other proven/probable fungal etiologies (n = 33) has been excluded.

^f Includes 197 proven/probable IA cases and 2385 controls where results are based on pooled performance data [10]. Performance in relation to the detection of other proven/probable fungal etiologies (n = 352) has been excluded.

^g Includes 322 proven/probable IA cases and 3290 controls, and results are based on pooled performance data [11]. Performance in relation to the detection of other proven/probable fungal etiologies (n = 590) has been excluded.

^h Includes 252 proven/probable IA cases and 2410 controls, and results are based on pooled performance data [12]. Performance in relation to the detection of other proven/probable fungal etiologies (n = 650) has been excluded.

ⁱ Includes 245 proven/probable IA cases and 1063 controls, determined by combining case/control and PCR positivity data as described in Table 2 of the meta-analysis by Tuon [15].

^j Includes 263 proven/probable IA cases and 927 controls where performance was reported using a single positive PCR as significant. The results represent pooled parameters (sensitivity/specificity) and differ from the published data that were summary estimates as calculated by SROC analysis (sensitivity 91% and specificity 92%) [17].

^k Includes 319 proven/probable IA cases and 1266 controls where performance was reported using a single positive PCR as significant. The results represent pooled parameters (sensitivity/specificity) and differ from the published data that were summary estimates as calculated by SROC analysis (sensitivity 90.2% and specificity 96.4%) [18].

^l Includes 230 proven/probable IA cases and 1386 controls where performance was reported using a single positive PCR as significant [16].

^m Includes 374 proven/probable IA episodes and 1863 unlikely IA episodes, determined by combining the episode data as described in Table 1 (characteristics of studies) with the final study determination as described in Table 2 (quality assessment and final determination of inclusion vs exclusion based on QUADAS 2 tool), as published in the meta-analysis of Arvanitis et al [19].

Table 4. Comparison of the Performance of the Fungitell, Fungitec G-Test, and Wako β -D-Glucan Assays for the Detection of Invasive Aspergillosis Preinclusion Into the European Organisation for the Research and Treatment of Cancer/Mycoses Study Group Definitions for the Diagnosis of Invasive Fungal Disease

| Performance Parameter | β -D-Glucan Assay | | |
|-------------------------|-------------------------|------------------|------------------|
| | Fungitell | Fungitec G-Test | Wako |
| No. of studies | 4 | 6 | 3 |
| Total population | 527 | 659 | 237 |
| Cases of IA (incidence) | 66 (12.5%) | 45 (6.8%) | 32 (13.5) |
| Sensitivity (95% CI) | 82% (70.9–89.3) | 80% (66.2–89.1) | 63% (42.5–77.1) |
| Specificity (95% CI) | 89% (86.2–91.9) | 87% (84.1–89.4) | 97% (93.1–98.3) |
| PPV (95% CI) | 52.4 (39.9–64.7) | 31.0 (21.1–43.0) | 74.1 (48.2–90.2) |
| NPV (95% CI) | 97.2 (94.2–98.7) | 98.3 (96.2–99.3) | 94.3 (88.6–97.3) |
| Positive LR | 7.70 | 6.14 | 18.30 |
| Negative LR | 0.20 | 0.23 | 0.39 |
| DOR | 37.8 | 26.7 | 47.1 |

Abbreviations: CI, confidence interval; DOR, diagnostic odds ratio; IA, invasive aspergillosis; LR, likelihood ratio; NPV, negative predictive value; PPV, positive predictive value.

Seven retrospective studies comprised 560 patients with an IA incidence of 14.5% [54, 55, 57–60]. Six prospective studies comprised 863 patients with an IA incidence of 7.2% [53, 61–65]. The difference between prospective and retrospective sensitivity was significant ($P = .0093$) (Table 2), although the proportion of cases tested by the different BDG assays varied between retrospective and prospective testing ($P = .0287$). In retrospective testing, 54.3% of cases were tested by the Fungitell assay, compared with 35.5% during prospective testing.

A comparison of performance of various BDG assays for the detection of IA is shown in Table 4. The sensitivity of the Fungitell was significantly greater than that of the Wako assay ($P = .0469$), whereas the specificity and positive predictive values of the Wako assay were significantly higher than both Fungitec G-Test and Fungitell assays ($P < .0001$ and $P = .0014$, respectively).

All testing was performed on serum or plasma samples, with no pediatric data available.

Characteristics and Clinical Utility of β -D-Glucan Before 2008

With 4 different commercial assays, available method variability was evident (Table 1 and Supplementary Table 1). In addition, positivity thresholds varied and were still to be finalized for individual assays. The BDG assay is capable of detecting a broad range of fungi, although it is not clear whether a single threshold for positivity is applicable to all etiologies [66]. No QC programs were available to monitor performance. Both false-positive and false-negative results were observed (Supplementary Table 1).

From a strategic viewpoint, twice-weekly testing was strongly advocated. The utility of BDG positivity on patient management was limited; some studies showed it to be an early indicator of disease, whereas others thought it had limited impact on patient management, although combining its use with other

diagnostics (high resolution computed tomography, GM-EIA or PCR) could be beneficial (Supplementary Table 1). No strategic studies investigating utility were available.

EVIDENCE FOR MOLECULAR-BASED ASSAYS

Comparison of *Aspergillus* PCR With GM-EIA and β -D-Glucan Methodological Standardization

PCR was excluded from EORTC/MSG definitions due to the lack of a standard method and associated clinical validity. However, overall clinical performance as determined by meta-analysis of the various methods available is comparable to that of GM-EIA and BDG (Table 3). Performance aside, standardized methodology provides intra and interassay consistency, QC, and a standardized approach for the diagnosis of IA.

Over the past decade, national attempts have been made to standardize fungal PCR [67, 68]. Internationally, the EAPCRI has, through the distribution of blinded QC panels, identified that DNA extraction, not PCR amplification, was the critical factor, and provided recommendations for *Aspergillus* DNA extraction from ethylenediaminetetraacetic acid blood, serum, and plasma [69–71]. Several commercial PCR assays capable of detecting *Aspergillus* now exist [72–77]. Most provide a standardized PCR amplification system that when combined with EAPCRI recommendations provide a fully standardized approach.

Control Material

Any commercial PCR assay will provide material to control performance. Additionally, PCR has the advantage of an independently developed and internationally standardized *Aspergillus* DNA calibrator, used to compare PCR amplification performance between centers [78]. This calibrator could be used to develop an

international standard material, permitting comparison of the entire molecular process and bringing *Aspergillus* PCR in line with other molecular diagnostic assays (human immunodeficiency virus, hepatitis C virus, or cytomegalovirus).

Quality Control

Neither GM-EIA nor BDG had QC schemes when incorporated into EORTC/MSG definitions. Currently, external QC for *Aspergillus* PCR can be independently determined by testing the Quality Control for Molecular Diagnostics *Aspergillus* species DNA panel. The EAPCRI has responded to user feedback and is providing performance information for individual centers for QC purposes.

Clinical Validity

PCR Testing of Blood and BAL Samples: Clinical Considerations. Although both blood and BAL fluid are regularly tested by PCR assays, their utility differs considerably. Clinical evidence consistent with IA may prompt a diagnostic workup involving BAL fluid, which, if positive, would improve confidence in the diagnosis when assay specificity is high. Conversely, the testing of multiple blood samples, as part of a screening strategy, increases sensitivity sufficiently, such that consistently negative tests results exclude disease. The 2 approaches are not mutually exclusive and should be used to complement each other, with frequent positivity during screening or clinical deterioration prompting a diagnostic workup.

From a diagnostic perspective, the specificities of PCR BAL testing were significantly greater than when testing blood by PCR ($P < .0001$; Table 3). Conversely, when screening, the sensitivity when testing blood as determined by Mengoli et al [16] was significantly greater than BAL PCR ($P = .0012-.015$). This was also true for the study of Arvanitis et al [19] when blood was compared to the BAL fluid study of Avni et al [18] ($P = .02$).

Comparison of PCR With Antigen Detection When Testing Blood. Although the specificity of PCR (Table 3) was significantly lower ($P < .0001$) than was found for GM-EIA and BDG before their inclusion into EORTC/MSG definitions (Table 2), the PCR sensitivity, as determined by the study of Mengoli et al [16] was significantly higher than both GM-EIA ($P = .048$) and BDG ($P = .009$). When comparing meta-analyses, the specificity of both antigen tests remained significantly higher than for PCR ($P < .0001-.012$), whereas the sensitivity of PCR remained significantly higher than for BDG ($P < .0001-.0477$).

Comparison of PCR With Antigen Detection When Testing BAL. There was no significant difference in sensitivity ($P = .26-.38$) or specificity ($P = .74-.76$) of PCR compared with GM-EIA when testing BAL fluid before inclusion into the EORTC/MSG definitions. However, when comparing the meta-analyses of both assays, PCR consistently had higher specificity ($P < .0001-.0019$; Table 3).

Further Considerations. *Aspergillus* PCR testing using methods consistent with the EAPCRI recommendations was associated with significantly greater specificity (98%) over non-compliant protocols (85%), coupled with a trend toward improved sensitivity (87%) for compliant over noncompliant methods (82%), providing evidence for the benefits of standardization [19]. Further methodological standardization can be achieved by automated testing of serum/plasma, providing performance comparable to that of whole blood without complex DNA extraction procedures [79].

False positivity is usually individual to that assay (for GM-EIA and BDG, see Supplementary Table 1). Fungal PCR false positivity has been associated with molecular biology reagents, including manual and automated extraction platforms, but also Vacutainer contamination, nutritional supplements, and respiratory tract colonization [80–87]. Both false-positive and false-negative results can occur through the use of inappropriately designed oligonucleotides, particularly if these interact with human DNA [67, 82]. Like other biomarker assays, false-negative results can occur through the prior use of antifungal agents for prophylaxis or therapy [88].

Assay Characteristics and Clinical Utility. Recently, a multicenter randomized controlled trial comparing nonculture diagnostics (GM-EIA/PCR) with culture and histology showed that the nonculture arm improved diagnosis and significantly reduced the use of empiric antifungal therapy (17%; $P = .002$), with no significant effect on all-cause or fungal-related mortality. No cases of IA were missed in the nonculture arm, providing an effective strategy for managing IA [89]. In a further multicenter randomized study, there was an earlier time to diagnosis, reduced use of empirical antifungal therapy, and reduced diagnoses of proven and probable IA, as well as improved fungal-free survival using combined PCR/GM-EIA surveillance compared with GM-EIA alone [90].

A reduction in unnecessary antifungal therapy was also shown in a prospective cohort study evaluating routine nonculture screening of 549 hematological patients [91]. Over a 5-year period, antifungal expenditure was approximately halved. The combined PCR/GM-EIA strategy had a sensitivity of 98.1%, and consistently negative tests could be used to exclude IA (likelihood ratio of a negative test result, 0.04). If both tests were positive on multiple occasions, a positive likelihood of 11.16 was highly suggestive of disease. Combining both tests showed optimal clinical utility for the diagnosis and management of IA.

With GM-EIA cross-reacting with other fungal species [20, 92], and BDG detecting a broad range of fungi, PCR is the only non-culture-based assay that can be designed to identify *Aspergillus* to a species level providing accurate epidemiology, and identifying species that may prove resistant to antifungal drugs (eg, amphotericin B vs *Aspergillus terreus* or *Aspergillus lentulus*).

Table 5. Summary of Currently Available Assay Characteristics of Biomarker Assays Capable of Detecting Invasive Aspergillosis With an Impact on Clinical Use

| Characteristic | GM-EIA to Date | β -D-Glucan to Date | PCR to Date |
|------------------------------------|--|--|---|
| Methodological recommendations | Single commercial assay with SOP: Platelia <i>Aspergillus</i> antigen (BioRad) | Five different commercial assays available utilizing different methods and materials: Fungitell (Associates of Cape Cod) Fungitec G-Test MK (Seikagaku Corporation) B-G Star (Maruha Corporation) (limited clinical validation reported) B-Glucan Test Wako (Wako Pure Chemicals) Dynamiker Fungus (1–3)- β -D-Glucan Assay (Dynamiker Biotechnology (Tianjin) Co, Ltd (limited clinical validation reported)) | Several commercial assays: Pathonostics Aspergenius Roche Septifast Myconostica MycAssay Ademtech Mycogenie Renishaw Fungiplex Procedural recommendations for DNA extraction (EAPCRI) |
| Control material | Controls supplied by the manufacturer No independent material available | Controls supplied by the manufacturer No independent material available | Controls supplied by the manufacturer International DNA calibrator available |
| Quality control | Internal – BioRad Proficiency panel | No | Independent – QCMD and EAPCRI panels |
| Sensitivity range ^a , % | Blood: 79.3 BAL: 83.6–85.7 | Blood: IA: 56.8–77.1 | Blood: 84–88 BAL: 76.8–79.6 |
| Specificity range ^a , % | Blood: 80.5–86.3 BAL: 89.0–89.4 | Blood: 81.3–97.0 | Blood: 75–76 BAL: 93.7–94.5 |
| False positives | Yes | Yes | Yes |
| False negatives | Yes | Yes | Yes |
| Clinical utility | Yes ^{b,c,d} | No | Yes ^{b,c,d} |

Abbreviations: BAL, bronchoalveolar lavage; EAPCRI, European *Aspergillus* PCR initiative; GM-EIA, galactomannan enzyme immunoassay; PCR, polymerase chain reaction; QCMD, Quality Control for Molecular Diagnostics; SOP, standard operating procedure.

^a Range determined from results presented in the relevant meta-analyses (see Table 3).

^b Morrissey et al [89].

^c Aguado et al [90].

^d Barnes et al [91].

Azole resistance in *A. fumigatus* is also a potential clinical concern; molecular-based assays can detect specific mutations that confer resistance [73, 93]. Direct testing of a clinical sample will improve sensitivity and reduce the time to result compared with culture.

CONCLUSIONS

The development of methodological recommendations together with commercially available assays allows PCR to be standardized, and potentially associated with improved performance.

In terms of assuring the quality and consistency of assay performance, only PCR has independently available control material and international QC schemes to calibrate and allow impartial comparison of different assays.

The diagnostic odds ratio for PCR is comparable to that for GM-EIA and BDG. The specificity of BAL PCR is greater than GM-EIA, providing better utility for a diagnostic test. When testing serum, the sensitivity appears superior for PCR, making it more suited to a screening strategy. Although sources of false positivity and negativity may be peculiar to PCR, the associated risks are similar to other biomarkers.

The revised EORTC/MSG definitions emphasize that the only difference between probable IA and possible IA is the presence or absence of mycological evidence of *Aspergillus*. It is therefore important that the analytical specificity of any assay is sound. Currently, PCR is the only nonculture assay that has the capability of being genus and species specific, and has the added potential to determine azole resistance.

The use of a standardized PCR may improve performance, and recent evidence suggests that testing in combination with GM-EIA may provide the optimal management strategy; future definitions should emphasize this. A comprehensive evaluation of PCR is required to overcome the limitations associated with this current review of selected articles. A summary of the current status of biomarker testing is shown in Table 5, and expands the evidence base for antigen testing beyond the information limited by preincorporation into the EORTC/MSG definitions. The information available for PCR is at least comparable to that for other tests, and the significance of PCR positivity should be in line with other biomarkers. These findings are commended to the wider fungal community to ultimately decide whether PCR is sufficiently validated and standardized for inclusion in the EORTC/MSG definitions of invasive fungal disease.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (<http://cid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Note

Potential conflicts of interest. P. L. W. is a founding member of the European Aspergillus PCR Initiative (EAPCRI); received project funding from Myconostica, Luminex, and Renishaw Diagnostics; was sponsored by Myconostica, MSD, and Gilead Sciences to attend international meetings; and provided consultancy for Renishaw Diagnostics Limited. J. R. W. has received speaker fees from Pfizer and has provided consultancy for Merck, Astellas, Ansun and Gilead. S. B. is a founding member of the EAPCRI, received project funding from Renishaw Diagnostics, was sponsored by Pfizer and MSD to attend international meetings, and provided consultancy for Gilead. J. L. is a founding member of the EAPCRI, received an educational grant and scientific fellowship award from Pfizer, and was sponsored by Astellas to attend international meetings. T. F. P. has received research grants from Astellas and Merck, and has been a consultant or scientific advisory board member for Astellas, Merck, Scynexis, Toyoma, and Viamet. M. A. S. has been a member of advisory boards for and received research funding from Pfizer, MSD, Schering-Plough, and Gilead Sciences; serves on the Antifungal Advisory Boards of Gilead Sciences Inc, MSD, and Pfizer; and has received funding in the form of untied grants from Gilead Sciences Inc, MSD Australia, and Pfizer Australia. R. A. B. is a founding member of the EAPCRI, received an educational grant and scientific fellowship award from Gilead Sciences and Pfizer, is a member of the advisory board and speakers' bureau for Gilead Sciences, MSD, Astellas, and Pfizer, and was sponsored by Gilead Sciences and Pfizer to attend international meetings. P. G. P. has received research support from Astellas, Merck, Gilead, Scynexis, and T2 Biosystems; and has served as an ad hoc scientific advisor for Astellas, Merck, Gilead, Scynexis, T2 Biosystems, and Viamet. J. P. D. is a founding member of the EAPCRI, is a member of the advisory board for Gilead Sciences and Pfizer, and has been on speakers' bureaus for Gilead Sciences, MSD, and Pfizer.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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